CLASS: I B.Sc Biotechology **COURSE NAME: General Microbiology Practical**
COURSE CODE: 18BTU213 **SEMESTER II** **BATCH: 2018-2021**

4H - 2C Total hours/week: L:0 T:0 P:3 **Marks: Internal: 40**
External: 60 Total: 100

Syllabus

1. Preparation of media & sterilization methods
2. Methods of Isolation of bacteria from different sources.
3. Staining methods: simple staining, Gram staining, spore staining, negative staining, hanging drop.
4. Biochemical characterization of isolated microbes.
5. Enumeration of microorganism - total & viable count.
6. Determination of bacterial cell size by micrometry.

References

1. Brooks, G.F, Carroll, K.C., Butel, J.S., & Morse, S.A. (2007). Jawetz, Melnick and Adelberg's *Medical Microbiology* (24th ed.). McGraw Hill Publication.

2. Goering, R., Dockrell, H., Zuckerman, M., & Wakelin, D. (2007). *Mims' Medical Microbiology* (4th ed.). Elsevier.

3. Willey, J.M., Sherwood, L.M., & Woolverton, C.J. (2008). *Prescott, Harley and Klein's Microbiology* (7th ed.). McGraw Hill Higher Education

SEMESTER II

18BTU213 GENERAL MICROBIOLOGY PRACTICAL 4H - 2C Total hours/week: L:0 T:0 P:3 Marks: Internal: 40 External: 60 Total: 100

Practical

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2. Methods of Isolation of bacteria from different sources.
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1. PREPARATION OF MEDIA

Introduction

Microorganisms in nature grow everywhere. But under lab conditions they can e grown if optimal conditions are provided. Culture medium is a material that fulfills the nutritional requirements of microorganisms. The selection of medium depends upon the kind of microorganism to be grown.

Aim

To study the preparation of culture media.

Materials required

Reagents: Components of media,

Glassware: Conical flask, glass rod, petriplate, test tubes

Equipments: balance, autoclave, etc.

Background

Microorganisms like all other living organisms requires basic nutrient for sustaining life. The food material on which microorganisms grow under laboratory conditions is known as culture media or in the other words we can define it as material that fulfills the nutritional requirement of microorganisms. Although all microorganisms have the same basic, requirement but there is diversity in terms of organic or inorganic compounds. Thus culture media vary in form and composition depends on the species to be cultivated.

Classification based on nutrition

a) Complex media or non – synthetic media:

The media which contain ingredients of comprise composition are called as complex media. Complex media are rich in the type and concentration of nutrients. They contain water- soluble extracts from partially degraded plant or animal tissue. By extract, yeast extract and peptone in the nutrient broth are undefined in composition.

These extracts are abundant source of amino acids, sugar, vitamins and minerals. Frequently, a sugar such as glucose is added to the peptone, tryptone or yeast extract to serve as a main carbon and energy source in the complex medium.

b) Defined media or synthetic media:

These media are generally from chemical compounds that are highly purified and precisely defined. Such media are reproducible.

The organism growing in defines medium can synthesis all their polysaccharides, protein, nucleic acid and lipids needed for growth from a single sugar, glucose as its carbon and energy, an inorganic nitrogen source (ammonium sulphate) and various mineral salts. More fastidious bacteria require the addition of known qualities as growth factors. For the growth of microorganism‘s liquid, solid and semi-solid media can be used.

Classification based on the physical state of medium

Agar-Agar is that component of the medium that helps in the solidification of the liquid medium (broth). It is also known as agar. It is a complex polysaccharide consists of 3,6 – unhydro – L – galactose and D – galatopyranose, it is also pure from nitrogen source. It is easily and chiefly extracted from red alga (seaweed) belonging to genera Grassilaria, Gelidium, etc.

a) Solid medium

It contains 1 to 2 % agar agar that helps in the solidification of media. It provides a solid surface for microorganisms to grow. For example nutrient agar medium, potato-dextrose agar medium, yeast extract medium, etc.

b) Semi solid medium

It contains less than 1% agar agar and appears like jelly or provide semi solid surface. It is used to determine the mobility of some motile bacteria during growth.

c) Liquid medium

It is devoid of agar-agar and remains in liquid state. It is also known as broth. This is used for the study of bacterial growth kinetics. For example, glucose broth, citrate broth, etc.

Procedure

Put the weighted amount of constituents in 500 ml distilled water except agar-agar.

Heat with agitation for dissolving constituents.

Make up the volume to 1000 ml or 1 liter.

Adjust the pH of the medium using pH papers or pH meter by either 1 N NaOH or 1 N HCl.

Add agar-agar to the media and mix well with heating. If liquid media (broth) is made then agar-agar is not added.

Dispense the media in conical flasks (not more the 2/3rd of the flask). Apply cotton plug to the neck of the flask.

Autoclave the media at 121^{0}C, 15 lbs pressure for 15 to 20 minutes.

Allow the media to cool and use it for the culture or cultivation of microorganisms

If not in use then stored at low temperature and dust free environment.

Safety guidelines

The cotton plug should be tight enough to restrict the entry of contaminated air.

Different constituents of media should be weighed accurately.

Adjust the pH to its optimal value.

Media should be sterilized accurately.

Store the media at low temperature for further use in a dust free environment.

B. STERILIZATION METHODS

Introduction

Sterilization is the use of a physical or chemical procedure to destroy all microbial life, including highly resistant bacterial endospores.

Decontamination is a term used to describe a process or treatment that renders a medical device, instrument, or environmental surface safe to handle. A decontamination procedure can range from sterilization to simple cleaning with soap and water. Sterilization, disinfection and antisepsis are all forms of decontamination.

Disinfection eliminates virtually all pathogenic non-spore-forming microorganisms but not necessarily all microbial forms on inanimate objects (work surfaces, equipment, etc.). Effectiveness is influenced by the kinds and numbers of organisms, the amount of organic matter, the object to be disinfected and chemical exposure time, temperature and concentration.

Antisepsis is the application of a liquid antimicrobial chemical to skin or living tissue to inhibit or destroy microorganisms. It includes swabbing an injection site on a person or animal and hand washing with germicial solutions.

Methods

There are four main categories of physical and chemical means of decontamination. They are heat, liquid disinfection, vapors and gases, and radiation.

1. Heat

Wet heat is the most dependable method of sterilization. Autoclaving (saturated steam under pressure of approximately 15 psi to achieve a chamber temperature of at least 250 degrees F for a prescribed time) rapidly achieves destruction of microorganisms, decontaminates infectious waste and sterilizes laboratory glassware, media, and reagents. For efficient heat transfer, steam must flush the air out of the autoclave chamber. Before using the autoclave, check the drain screen at the bottom of the chamber and clean it, if blocked. If the sieve is blocked with debris, a layer of air may form at the bottom of the autoclave, preventing efficient operation. Prevention of entrapment of air is critical to achieving sterility. Material to be sterilized must come in contact with steam and heat.

Chemical indicators, e.g., autoclave tape, must be used with each load placed in the autoclave. The use of autoclave tape alone is not an adequate monitor of efficacy. Autoclave sterility monitoring should be conducted on a regular basis (at least monthly) using appropriate biological indicators (*B.stearothermophilus* spore strips) placed at locations throughout the autoclave. The spores, which can survive 250 degrees F for 5 minutes but are killed at 250 degrees F in 13 minutes, are more resistant to heat than most, thereby providing an adequate safety margin when validating decontamination procedures. Each type of container employed should be spore tested because efficacy varies with the load, fluid volume, etc.

Decontaminate all infectious materials and all contaminated equipment or labware before washing, storage or discard as infectious waste. Autoclaving is the preferred method.

Dry heat is less efficient than wet heat and requires longer times and/or higher temperatures to achieve sterilization. It is suitable for the destruction of viable organisms on impermeable non-organic surfaces such as glass, but it is not reliable in the presence of shallow layers of organic or inorganic materials which may act as insulation. Sterilization of glassware by dry heat can usually be accomplished at 160-170 degrees C for periods of 2-4 hours. Dry heat sterilizers should be monitored on a regular basis using appropriate biological indicators [*B.subtilis (globigii)* spore strips].

Incineration is another effective means of decontamination by heat. As a disposal method, incineration has the advantage of reducing the volume of the material prior to its final disposal.

2. Liquid Disinfection

The most practical use of liquid disinfectants is for surface decontamination and, when used in sufficient concentration, as a decontaminate for liquid wastes prior to final disposal in the sanitary sewer. If liquid disinfectants are used, they must have been shown to be effective against the organism(s) present.

Liquid disinfectant are available under a wide variety of trade names. In general, these can be classified as: halogens, acids, alkalis, heavy metal salts, quaternary ammonium compounds, phenolic compounds, aldehydes, ketones, alcohols and amines. The more active a compound is, the more likely it is to have undesirable characteristics such as corrosivity. No liquid disinfectant is equally useful or effective under all conditions and for all viable agents.

3. Vapors and Gases

A variety of vapors and gases possess decontamination properties. Vapors and gases are primarily used to decontaminate biological safety cabinets and associated systems, bulky or stationary equipment not suited to liquid disinfectants, instruments or optics which might be damaged by other decontamination methods, and rooms, buildings and associated air-handling systems. Agents included in this category are glutaraldehyde and formaldehyde vapor, ethylene oxide gas, peracetic acid and hydrogen peroxide vapor.

When used in closed systems and under controlled conditions of temperature and humidity, excellent disinfection can be obtained. Great care must be taken during use because of the hazardous nature of many of these compounds. Vapor and gas decontamination should not be attempted by research personnel.

4. Radiation

Although ionizing radiation will destroy microorganisms, it is not a practical tool for laboratory use. Non-ionizing radiation in the form of ultraviolet radiation (UV) is used for inactivating viruses, bacteria and fungi. It will destroy airborne microorganisms and inactivate microorganisms on exposed surfaces or in the presence of products of unstable composition that cannot be treated by conventional means.

Because of the low penetrating power of UV, microorganisms inside dust or soil particles will be protected from its action, limiting its usefulness. UV is used in air locks, animal holding areas, ventilated cabinets and laboratory rooms to reduce levels of airborne microorganisms and maintain good air hygiene. Because UV can cause burns to the eyes and skin of people exposed for even a short period of time, proper shielding should be maintained when it is in use. UV lamps that are used for space decontamination should be interlocked with the general room or cabinet illumination, so that turning on the lights extinguishes the UV.

Result:

Glass wares and media that are necessary for cultivation of microbes were sterilized.

2. METHODS OF ISOLATION OF BACTERIA FROM DIFFERENT SOURCES

A. Isolation of microorganisms from soil

Aim

To isolate the microorganisms from soil sample.

Principles

Soil is the principle habitat for a variety of microorganism. The Quality and quantity of microbes depend upon the physico-chemical characteristics of soil. Enrichment media promote the growth of a particular microorganism against the others present in the sample by its specific nutrient utilization ability or other unique metabolic properties (e.g. resistance to antibiotics or heavy metals). Only those microbes can grow in the enrichment medium that can use or tolerate the components of the selective medium.

Materials required

Soil sample, nutrient agar, sterile distilled water, petridish, test tubes, conical flask, colony counter.

Procedure

1. Measure 1-1 g of the garden soil, 1ml of water into the flasks containing different enrichment broths and the preprepared nutrient agar plates directly exposed to air.
2. For better aeration, place the inoculated flasks into a shaker incubator at 28°C for one week.
3. Prepare a five-member, 10-fold dilution series from the enrichment cultures and the original sample.
4. Spread the surface of agar plates having the same composition as the enrichment broths from the individual members of the dilution series.
5. Incubate the cultures at 28°C for one week.
6. Perform germ count estimations and colony morphology examinations after the incubation period.

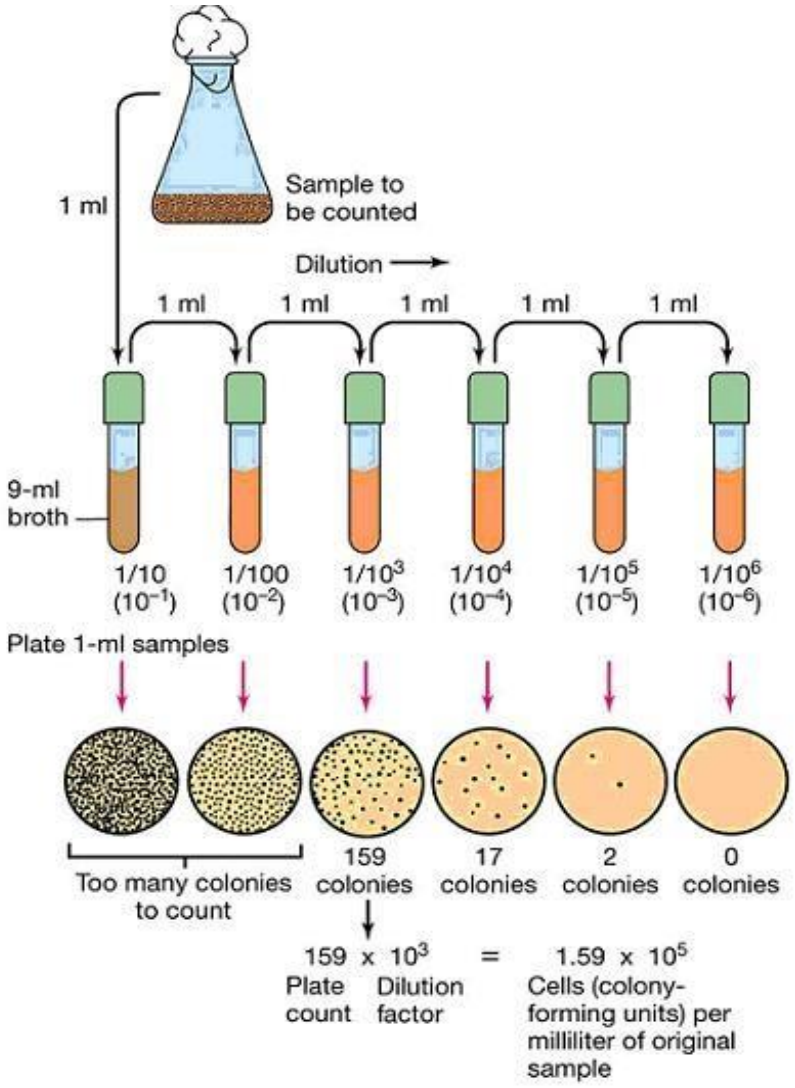
Calculations

Organism per ml of sample = Number of colonies / Amount plated x Dilution factor

Result

The amount of organism per ml of soil sample = ……..colonies.

Serial dilution methods



B. Isolation of microorganisms from water

Aim

To isolate the microorganisms from water sample

Principles

Polluted water is the principle habitat for a variety of microorganism. The Quality and quantity of microbes depend upon the physico-chemical characteristics of water. Enrichment media promote the growth of a particular microorganism against the others present in the sample by its specific nutrient utilization ability or other unique metabolic properties (e.g. resistance to antibiotics or heavy metals). Only those microbes can grow in the enrichment medium that can use or tolerate the components of the selective medium.

Materials required

Water sample, nutrient agar, sterile distilled water, petridish, test tubes, conical flask, colony counter.

Procedure

1. Measure 1-1 ml of the water sample and diluted with fresh water and pored into the flasks containing different enrichment broths and the preprepared nutrient agar plates directly exposed to air.
2. For better aeration, place the inoculated flasks into a shaker incubator at 28°C for one week.
3. Prepare a five-member, 10-fold dilution series from the enrichment cultures and the original sample.
4. Spread the surface of agar plates having the same composition as the enrichment broths from the individual members of the dilution series.
5. Incubate the cultures at 28°C for one week.
6. Perform germ count estimations and colony morphology examinations after the incubation period.

Calculations

Organism per ml of sample = Number of colonies / Amount plated x Dilution factor

Result

The amount of organism per ml of soil sample = ……..colonies.

C. Isolation of microorganisms from Air

Aim

To isolate the microorganisms from air

Principles

Air is the principle habitat for a variety of aerobic microorganism. The Quality and quantity of microbes depend upon the physico-chemical characteristics of air. Enrichment media promote the growth of a particular microorganism against the others present in the sample by its specific nutrient utilization ability or other unique metabolic properties (e.g. resistance to antibiotics or heavy metals). Only those microbes can grow in the enrichment medium that can use or tolerate the components of the selective medium.

Materials required

Nutrient agar, sterile distilled water, petridish, test tubes, conical flask, colony counter.

Procedure

1. The prepared nutrient agar plates were exposed to atmospheric air at different time intervals.
2. Incubate the cultures at 28°C for one week.
3. Perform germ count estimations and colony morphology examinations after the incubation period.

Result

The amount of organism was counted at various time interval of agar plate exposed to air.

D. Isolation of microorganisms from food

Aim

To isolate the microorganisms from food

Principles

Food is the principle habitat for a variety of microorganism. The Quality and quantity of microbes depend upon the physico-chemical characteristics of food. Enrichment media promote the growth of a particular microorganism against the others present in the sample by its specific nutrient utilization ability or other unique metabolic properties (e.g. resistance to antibiotics or heavy metals). Only those microbes can grow in the enrichment medium that can use or tolerate the components of the selective medium.

Materials required

Nutrient agar, sterile distilled water, petridish, test tubes, conical flask, colony counter.

Procedure

1. Measure 1-1 g of the food, 1ml of water into the flasks containing different enrichment broths and the prepared nutrient agar plates directly exposed to air.
2. For better aeration, place the inoculated flasks into a shaker incubator at 28°C for one week.
3. Prepare a five-member, 10-fold dilution series from the enrichment cultures and the original sample.
4. Spread the surface of agar plates having the same composition as the enrichment broths from the individual members of the dilution series.
5. Incubate the cultures at 28°C for one week.
6. Perform germ count estimations and colony morphology examinations after the incubation period

Calculations

Organism per ml of sample = Number of colonies / Amount plated X Dilution factor

Result

The amount of organism per ml of food sample = ……..colonies.

3. STAINING TECHNIQUES – SIMPLE STAINING, GRAM STAINING, SPORE STAINING, NEGATIVE STAINING AND HANGING DROP

STAINING TECHNIQUES

Objective

To learn the various staining techniques to study morphology of microorganisms.

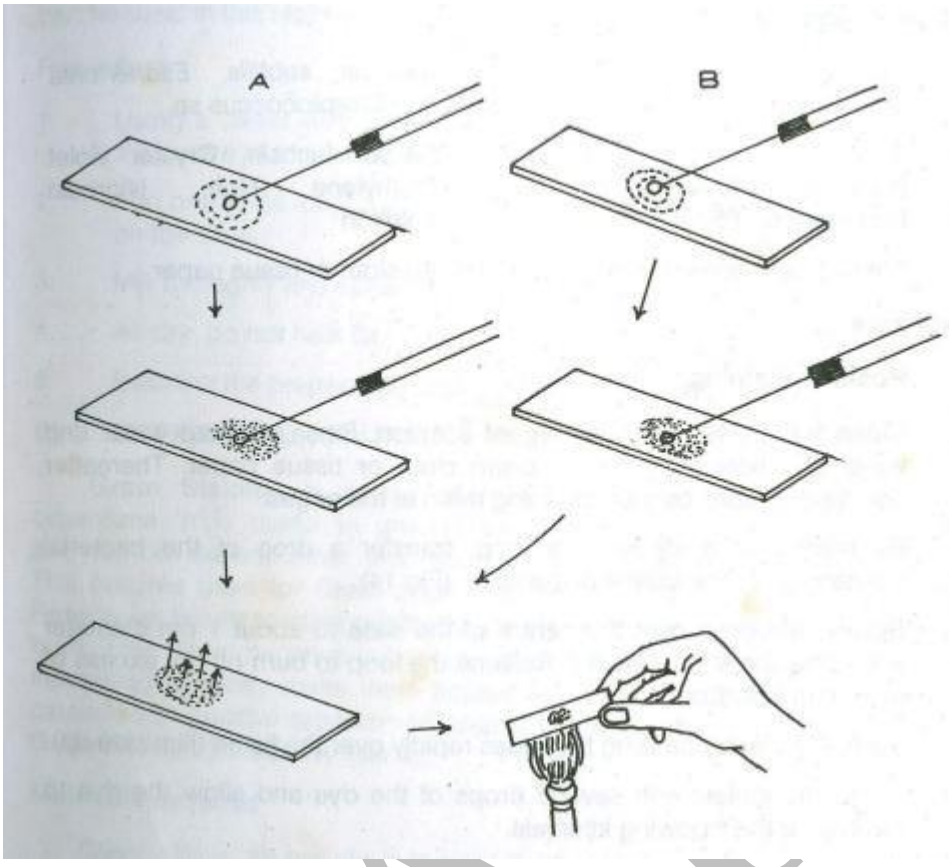
Background

Staining is the method of artificially producing colour in micorogranisms to allow for the visualization under the microscope; Stains are employed not only to make the organisms visible but also understand their structure and chemical nature.

Dyes or stains are synthetic chemical products of the aniline type. They can be divided into acid dyes and basic dyes with respect to their colour property in the anionic and cationic form. An acid dye is the salt of a coloured acid while a basic dye is the salt of a coloured base. For example, Crystal violet, Methylene blue are basic dyes and Acid fuchsin and Erythrosin are acid dyes. Acid dyes have stronger tendency to combine with the cytoplasm whereas basic dyes have greater affinity for the nuclear region of the cells. Basic dyes are generally used in bacteriological studies.

Knowledge of the basis for the specificity of dyes is important to understand differential staining reactions. For example in Gram's staining, the primary dyes are of the basic triphenyl methane type (crystal violet or methyl violet). Alkaline aqueous solution of these dyes readily penetrate the bacterial cells or yeast and stain them dark blue. If the stained cells are washed extensively with water or dilute acid much of the primary dye is removed. Instead, if the cells are next treated with iodine solution which acts as a mordant a water insoluble dye complex is produced. Then the cells are then treated with an organic solvent (alcohol or acetone) the dye iodine complex is quickly dissolved and tends to be removed from the cell' contents whereas blue colour persists longer in the cell walls of Gram positive bacteria.

Application of an aqueous counter stain results in staining the decolourlzed Gram negative cells whereas Gram positive cell wall do not react with the counter stain. Evidently the essential difference between Gram positive and Gram negative microorganisms is in the chemistry of the cell wall. However, some of the environmental factors can also influence the staining property of the cells. They are a) Age of the culture; b) pH of the environment (medium). The response to Gram Stain is of value in classifying bacteria.



A. Preparation of culture slide

B. From the liquid culture B. From the solid medium

A) SIMPLE STAINING

The simple stain contains only one dye that allows visualization of gross morphological characteristics of cells. With the use of this detailed differentiation is not possible.

Materials required

Cultures (24 hrs. old) : *Bacillus subtilis*, *Escherichia coli*, *Streptococcus sp*.

Stains : Carbol fuchsin, Crystal violet, Methylene blue, Nigrosin solution

Clean glass slides, Microscope and clean cloth or tissue paper.

Procedure

I. Positive Staining

1. Clean the slides in any detergent solution. Rinse with tap water and wipe the slides. Dry with a clean cloth or tissue paper. Thereafter handle the slides only by grasping them at the edges.
2. By means of a sterile culture loop, transfer a drop of the bacterial suspension to the centre of the slide.
3. Spread the drop over the centre of the slide to about 1 crn diameter and allow the film to air dry. Reflame the loop to burn off the excess of inoculam in it. Cool.
4. Fix the smear by passing the slides rapidly over the flame (film side up).
5. Flood the smear with several drops of the dye and allow the dye to remain for the following intervals.

   Carbolfuchsin - 15-30 sec.

   or

   Crystal violet - 30-45 sec.

   or

   Methylene Blue - about 3 mins.
6. Wash the slide in a gentle stream of tap water to remove excess of stain. Air dry.
7. Examine the preparation with the oil immersion objective.

OBSERVATION AND RESULT

B) GRAM STAINING

Gram Staining is employed to visualize and differentiate between organisms. It is useful in presumptive identification of organisms before carrying out several other tests such as seroloqlcal, and biochemical tests. The cultures used for Gram stain reaction should be less than 24 hr. old. Reason for failure to stain positively are age of culture, low pH of medium or both. True Gram negative organism will not become gram positive although increasing alkalinity make them appear as positive. Increase in acidity may cause Gram positive organism to appear Gram negative. Cocci are generally Gram positive (except Neisseria).

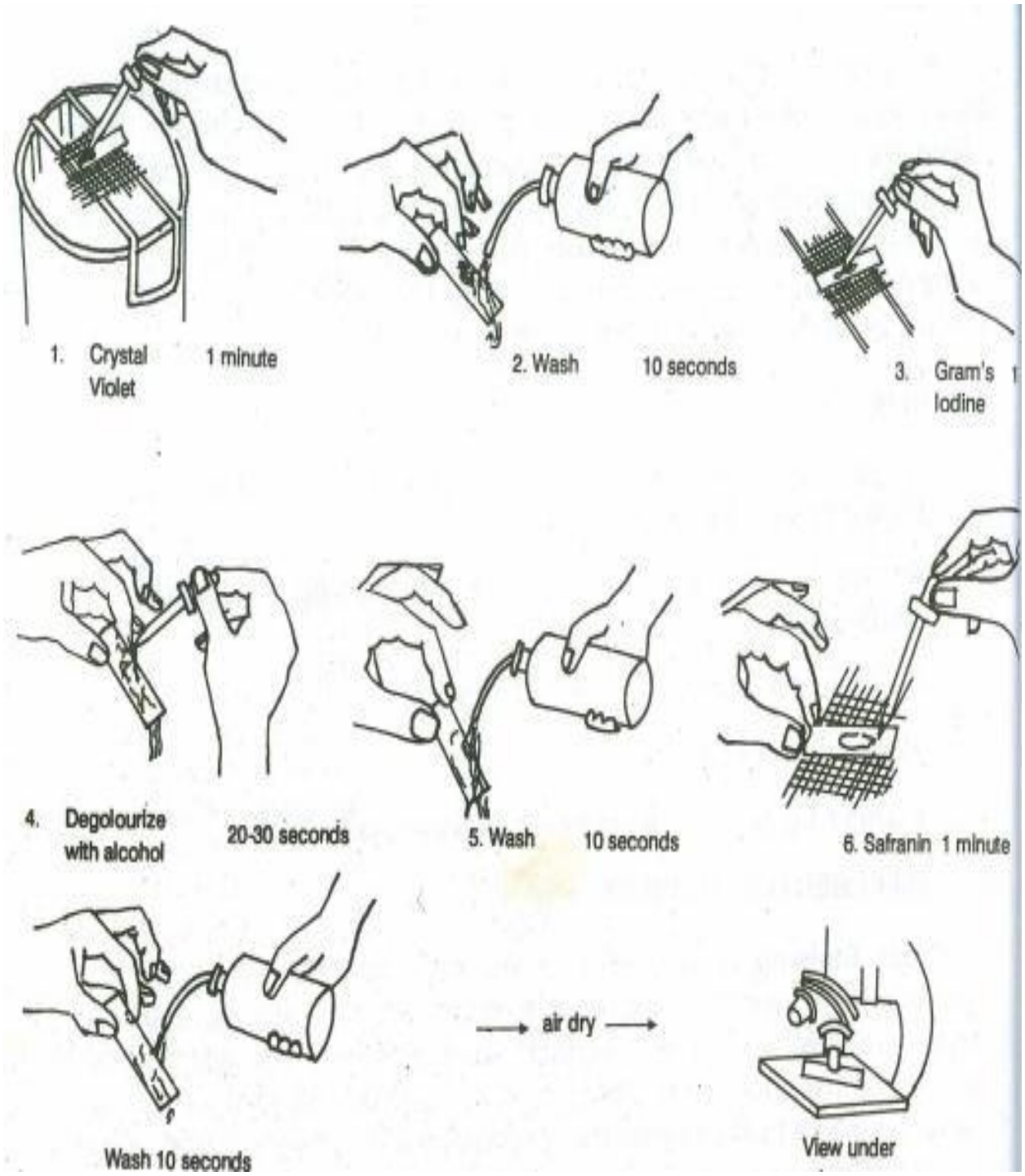
Materials required

Glass slides, 24 hrs old culture of Escherichia coli and Bacillus subtilis, Crystal violet, Potassium Iodide/Iodine Solution, Acetone-Alcohol, Safranin.

Procedure

1. Prepare the smear on the slides with the bacterial cultures as done for the simple positive staining method.
2. Stain it for one minute with crystal violet solution (Gram's stain)wash it in tap water.
3. Apply the Iodine solution (Mordant) for 1 min. Wash in tap water.
4. Decolourize with alcohol by adding dropwise on the tilted slide until all free blue colour has been removed (20-30 sec). Wash it in tap water.
5. Flood the slide with safranin (counter stain) for one minute. Wash it in tap water and air dry.
6. Examine the stained smear under the oil immersion objective to determine which organism is Gram positive (Violet colour) and which is Gram negative (Pink colour).

OBSERVATION AND RESULT



Microscope

Gram Staining

C) SPORE STAINING

Under conditions of inadequate nutrition and unfavourable for growth, specialized structures called spores or endospres are formed within certain Gram-positive cells such as aerobic rods (*Bacilli*) anaerobic rods (*Clostridia*) and a few cocci (*Sporosarcinae*). These spores have no metabolic activity and are resistant to heat, drying, freezing, toxic compounds and radiation. The spores have a high Ca + + content and "Dipicolinic acid" which forms 5-15% of their dry weight. Materials required Bacillus subtilis slants (24 hr. culture), Stain. a) Malachite green (5% w/v aqueous solution) and b) Mercurochrome (5% w/v aqueous solution).

Materials required

Bacillus subtilis slants (24 hr. culture), Stain. a) Malachite green (5% w/v aqueous solution) and b) Mercurochrome (5% w/v aqueous solution).

Procedure

1. Prepare a smear of *Bacillus subtilis* on a clean glass slide. Air dry and fix with heat.
2. Flood the slide with Malachite green and place a cut piece of paper to wetting over the smear, so that it completely covers the smear and soaks up most of the stain. The toweling should be saturated with the stain throughout the staining process.
3. Gently heat the slide until the stain begins to steam and keep it for 5 mins.
4. Remove the paper towel and wash the slide gently with water until all excess stain is removed.
5. Counter stain with basic fuchsin for 30 sec.
6. Wash with water. Blot it and air dry.
7. Examine the slide using oil immersion objective. Red colored cells containing green spores can be seen.

OBSERVATION AND RESULT

D) NEGATIVE STAINING

Advantage of this method is that there is less morphological distortion since cells are not subjected to vigorous physical or chemical treatment. Dyes utilized are acid and have colour associated with the negative ion. Bacterial cells are negatively charged. Therefore cell wall and dye have not affinity for each other and dye is deposited on the background i.e., around edges of cell. Hence this method is also referred to as indirect or "relief' staining. Dyes that can be used in this staining method are Nigrosin, India ink.

Procedure

1. Using a sterile wire loop, transfer a loopful of bacterial suspension to the centre of a clean slide.
2. Add one small loopful of nigrosin solution to the drop of the suspension on the slide.
3. Mix throughly and spread to form a thin film;
4. Air dry; do not heat fix.
5. Examine the preparation under the oil immersion objective.

OBSERVATION AND RESULT

E) HANGING DROP TECHNIQUE

Objective

To demonstrate the motility of bacterial cell.

Background

Heat fixing and staining are rather severe treatment for a bacterial cell and they cause considerable changes in morphology. To avoid these changes, bacteria can be observed in their living state by means of wet mount technique. In this method there is usually little difficulty with highly motile organisms but feebly motile organism may require prolonged observation of individual cells. Bacterial movement must be distinguished from "Brownian movement". Brownian movement is vibratory movement caused by invisible molecules striking the bacteria displacing them for short distance. True motility of the bacteria may lead to the bacteria to move from one place to other relatively longer distance. Therefore it is best to use hanging drop preparation for this purpose. For observing motility of pathogenic organism it is safer method is to inoculate semisolid medium and observe growth and motility.

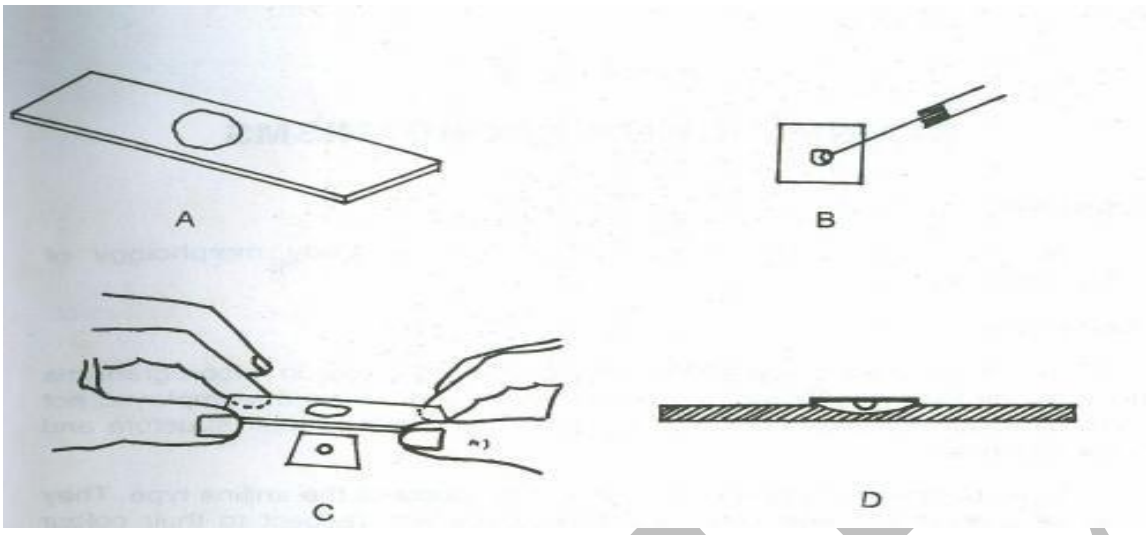
Materials required

A broth culture of motile bacteria, Proteus sp., Microscope, Single cavity slides, Coverslips, Vaseline, Soft agar medium.

Procedure

a) Hanging drop method

1. Place a very small drop of bacterial culture in the centre of a cover slip.
2. Place a small drop of vaseline at each corner of the depression side of the cavity slide.
3. Invert the slide (cavity side) over the cover slip. The cover slip adhere to the glass slide and when the cover slip is inverted,the hanging drop is suspended in the well.
4. Bring the edge of the hanging drop into the focus with 10X objective betore turning to the high power objective to observe motility.



Preparation of hanging drop

A. Cavity slide (vaselin applied over the edges)
B. Placing loopful organism at the centre of cover glass
C. Cavity slide placed over cover slip
D. Complete hanging drop slide for viewing

b) Stab culture method

1. Prepare the semisolid nutrient agar medium (0.7% agar) and distribute them in test tubes and sterilize them.
2. Allow the tubes to cool for 1 hr.
3. Sterilize the inoculation loop by flaming. Then cool it for a while.
4. Take a loopful of culture from the broth culture.
5. Remove the plug from the tube of semisolid medium, flame the mouth and stab medium with inoculation loop containing culture.
6. Flame the neck of the tube. Plug with the cotton.
7. Incubate for 48 h at 37°C in an incubator.
8. Examine growth of the bacteria in the medium in the tube. If the cloudiness appears indicate the motility of the bacteria. Non-motile organism grows only on the line of the stab.

OBSERVATION AND RESULT

4. IDENTIFICATION OF BIOCHEMICAL CHARECTERISTICS OF ISOLATED MICROBES

IMVIC TEST

Aim

The IMViC tests are used to differentiate the enterics (Family Enterobacteriaceae). These are the Indole test (tryptone broth), the Methyl Red and Voges-Proskauer tests (MRVP broth) and the Citrate test (Citrate agar slants). For these IMViC tests use the enterics *E. coli* and *Enterobacter.*

Materials

Culture of Escherichia coli, Culture of Klebsiella pneumoniae, SIM tubes, MR-VP broth tubes, Citrate agar slants, EMB Plates, Capped test tubes, nonsterile, 1 mL pipets, Pipettor, Inoculating loop, Bunsen burner, Striker, Kovac‘s reagent, Methyl red, pH indicator, Barritt‘s reagents A and B

1. Indole test (tryptone broth)

Procedure: Inoculate a loopful of bacteria into a tryptone broth. Incubate 48 hours.

Description: Tryptophan hydrolysis -Some bacteria split tryptophan into indole and pyruvic acid using the hydrolase called tryptophanase. Indole can be detected with Kovac's reagent (Indole reagent). This test is very important in differentiating *E. coli* (indole positive) from some closely related enteric bacteria. It also differentiates *Proteus mirabilis* (indole negative) from all other *Proteus* species (indole positive). Tryptone broth is used for this test as it contains a large amount of tryptophan.

Interpretation: After incubation: The broth must be turbid. A clear broth indicates that your organism did not grow and cannot be tested. Add a few drops of Indole reagent to the broth culture (tryptone broth). DO NOT SHAKE THE TUBE. A positive result has a red layer at the top. A negative result has a yellow or brown layer.

2. Methyl Red test (MRVP broth)

Procedure: Inoculate a loopful of bacteria into MRVP broth. Incubate 3 to 5 days.

Description: Mixed acid fermentation - Many gram-negative intestinal bacteria can be differentiated based on the products produced when they ferment the glucose in MR-VP

medium. *Escherichia*, *Salmone lla*, and *Proteus* ferment glucose to produce lactic, acetic, succinic, and formic acids and CO_2, H_2, and ethanol. The large amounts of acids produced lowers the pH of the medium - Methyl red (a pH indicator) will turn red when added to the medium if the organism was a mixed acid fermenter. Many of these organisms also produce gas. **Interpretation: After incubation**: The broth must be turbid. A clear broth indicates that your organism did not grow and cannot be tested. Remove 1 ml of broth and place into a sterile tube before performing the methyl red test if you are going to use the same broth for the VP test. Add

3-4 drops of methyl red to the original broth. DO NOT SHAKE THE TUBE. A positive result has a distinct red layer at the top of the broth. A negative result has a yellow layer.

3. Voges-Proskauer test

Procedure: Inoculate a loopful of bacteria into MRVP broth. Incubate 3 to 5 days.

Description: Organisms that are negative in the methyl red test may be producing 2, 3 butanediol and ethanol instead of acids. These non-acid products do not lower the pH as much as acids do. *Enterobacter, Serratia* and some species of *Bacillus* produce these substances. There is no satisfactory test for determining production of 2, 3 butanediol. A precursor of 2,3 butanediol called acetoin can be detected with Barritt's reagent.

Interpretation: After incubation: Read the VP test when you have good turbidity. A clear broth indicates that your organism did not grow and cannot be tested. Barritt's reagent A (VP A) contains naphthol and Barritt's B (VP B) contains KOH. Test 1 ml of your culture from the MRVP broth. If you have already conducted the methyl red test, you should have already placed

1 ml of untested broth in a sterile tube. If you haven‘t done this, do so now. Add the entire contents of the VP A reagent (15 drops) and 5 drops of the VP B reagent to the 1 ml of your broth culture. SHAKE WELL. This reaction will take a few minutes before you will see a color change. SHAKE the tube every few minutes for best results. With a positive reaction the medium will change to pink or red indicating that acetoin is present. With a negative reaction the broth will not change color or will be copper colored. Wait at least 15 minutes for color to develop before calling the test negative.

4. Citrate test (Simmon's Citrate slant)

Procedure: Streak a loopful of bacteria onto a citrate agar slant, do *not* stab the butt. Incubate 24 to 48 hours, longer for *Bacillus* species. Incubate with a loose cap.

Description: Simmon's citrate agar tests for the ability of an organism to use citrate as its sole source of carbon. This media contains a pH indicator called bromthymol blue. The agar media changes from green to blue at an alkaline pH.

Interpretation: After incubation: A positive reaction is indicated by a slant with a Prussian blue color. A negative slant will have no growth of bacteria and will remain green.

UREASE TEST

Aim

To determine the ability of microorganisms to degrade urea by means of the enzyme urease.

Principle

Urea is a nitrogen containing compound that is produced during decarboxylation of the amino acid arginine in the urea cycle. Urea is highly soluble in water and is therefore an efficient way for the human body to discharge excess nitrogen. This excess urea is then taken out of the body through the kidneys as a component of urine. Some bacteria have the ability to produce an enzyme urease as part of its metabolism to break down urea to ammonia and Carbon dioxide.

Materials Required

Cultures

24-48 hours tryptic soy broth cultures.

Media

Urea broth
Urea slant

Equipments

Bunsen burner
Inoculating loop
Test tubes
Marking pen

Media Preparation

Urea test broth is prepared by adding 20 g of urea, 9.5 g of Na2HPO4, 9.1 g of KH2PO4, 0.1g of yeast extract and 0.01 g of phenol red. The pH is made to 6.8±0.2 at 25°c.

Urea slants are prepared in the same way as the preparation of broth, but add agar also. Mix thoroughly and dispense aseptically in sterile tubes. Cool the tubed medium in a slanted position so that deep butts are formed.

Procedure

Using a sterile technique, inoculate each experimental organism into its appropriately labeled tube by means of loop inoculation. Incubate cultures 24-48 hours at 37°C.

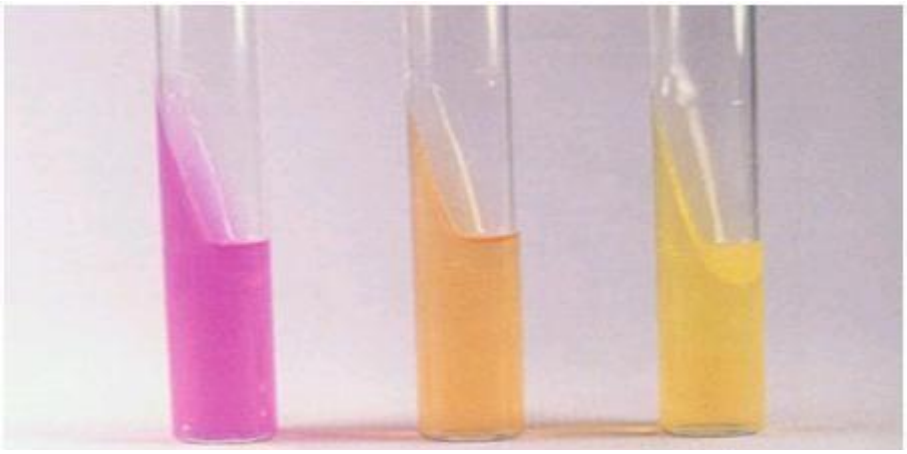
a) Urea Broth method

1. Sterilize the loop in the blue flame of the Bunsen burner till red hot. Heat from the base of the wire first and slowly move towards the loop (tip). Heat the wire until it is red-hot and then allowed to cool.
2. Take out a loopful organism from the tryptic broth culture tube with the cooled loop aseptically.
3. Again flame the neck of the tube and replace the tube in the test tube rack.
4. Take a sterile urea broth tube, remove the cap and flame the neck of the tube.
5. Inoculate the urea broth with the inoculation loop containing the organism from the tryptic soy broth culture.
6. Again flame the neck of the urea tube and place it in the test tube rack.
7. Incubate for 24-48 hours at 37°C.
8. Obtain the broths from the incubator and observe the colour.

Uninoculated Positive Negative

b) Urea Slant method

1. Sterilize the loop in the blue flame of the Bunsen burner till red hot and then allowed to cool.
2. Take out a loopful organism from the tryptic broth culture tube with the cooled loop aseptically.
3. Again flame the neck of the tube and replace the tube in the test tube rack.
4. Take a sterile urea slant tube remove the cap and flame the neck of the tube.
5. Inoculate the entire surface of the urea slant (slope) with the provided growth from the tryptic broth culture using the inoculating loop (do not stab the butt). The slant of the medium is inoculated by streaking the surface of the agar in a zigzag manner.
6. Again flame the neck of the urea tube and place it in the test tube rack.
7. Tighten the cap and incubate at 37°C for 24-48 hours.
8. Obtain the tubes from the incubator and observe the colour change.



Positive Control Negative

CARBOHYDRATE FERMENTATION

Objective

To find the ability of microorganisms to ferment the given Carbohydrate.

To determine the ability to degrade amino acids.

To determine the ability of microorganism to produce gaseous end products in fermentation.

Principle

A metabolic process performed by almost all types of bacteria is known as fermentation. This will result in the production of ATP, the ultimate energy source of the organism. This will happen either in the presence or absence of atmospheric oxygen. Bacteria utilize the nutrients in their environment to produce ATP for their biological processes such as growth and reproduction. The enzyme systems in bacteria allow them to oxidize environmental nutrient sources. Bacteria will use different energy sources in the medium depends on the specific enzymes of each bacteria. Many bacteria possess the enzymes system required for the oxidation and utilization of the simple sugar, glucose. Some bacteria have the ability to degrade complex carbohydrates like lactose, sucrose or even polysaccharides. Such bacterium should possess the enzymes that should cleave the glycosidic bonds between the sugar units and the resulting simple carbohydrate can be transported into the cell. Lactose is a disaccharide consisting of the glucose and galactose connected by glycosidic bond. The bacteria which produce the enzyme lactase will

break this bond and thus release free glucose that can be easily utilized by the organism. The characteristics feature of the enzyme production in the bacteria enables them to use diverse carbohydrates and this will aid in the identification of unknown bacteria.

Materials Required

Phenol Red Carbohydrate Fermentation Broth.
Bacterial culture.
Inoculation loop.
Incubator (37^0 C).

Procedure

I. Preparation of Carbohydrate Fermentation Broth

1. Weigh and dissolve trypticase, Sodium chloride, and Phenol red in 100 ml distilled water and transfer into conical flasks.
2. Add 0.5% to 1% of desired carbohydrate into all flasks.
3. Insert inverted Durham tubes into all tubes, the Durham tubes should be fully filled with broth.
4. Sterilize at 115^0 C for 15 minutes.
5. **Important**: Do not overheat the Phenol red Carbohydrate fermentation broth. The overheating will result in breaking down of the molecules and form compounds with a characteristic color and flavour. The process is known as caramelization of sugar (the browning of sugar).
6. Transfer the sugar into screw capped tubes or fermentation tubes and label properly.

Ingredients of the Fermentation Broth:

1. Trypticase: 1g
2. Carbohydrate: 0.5g
3. Sodium Chloride: 0.5g
4. Phenol red : 0.0189mg

*Autoclave at 115^o C for 15 minutes

II. Inoculation of Bacterial Culture into the Phenol Red Carbohydrate Broth

Aseptically inoculate each labeled carbohydrate broth with bacterial culture.(keep uninoculated tubes as control tubes).

Incubate the tubes at 18-24 hours at 37^{o}C.

Observe the reaction.

Precautions:

After inoculation into a particular sugar, sterilize the loop in order to avoid cross contamination of the tube with other sugars.

Keep uninoculated sugar tubes as control tubes.

Do not use the tubes with Durham tubes that are partially filled or with bubbles.

Over incubation will help the bacteria to degrade proteins and will result give false positive results.

Expected Results

Results of carbohydrate fermentation test

1. **Acid production**: Changes the medium into yellow color- organism ferments the given carbohydrate and produce organic acids there by reducing the ph of the medium into acidic.
2. **Acid and Gas production**: Changes the medium into yellow color-organism ferments the given Carbohydrate and produce organic acids and gas. Gas production can be detected by the presence of small bubbles in the inverted durham tubes.
3. **Absence of fermentation**: The broth retains the red color. The organism cannot utilize the carbohydrate but the organism continues to grow in the medium using other energy sources in the medium.

Triple Sugar- Iron Agar Test

Objectives

1. To differentiate among members of the Enterobacteriaceae.
2. To distinguish between the gram-negative enteric bacilli from other groups of intestinal bacilli based on carbohydrate fermentation and the production of hydrogen sulfide.

Principle

The triple sugar- iron agar test is designed to differentiate among the different groups or genera of the Enterobacteriaceae, which are all gram negative bacilli capable of fermenting glucose with the production of acid, and to distinguish them from other gram negative intestinal bacilli. This differentiation is based on the differences in carbohydrate fermentation patterns and hydrogen sulfide production by the various groups of intestinal organisms. Carbohydrate fermentation is indicated by the presence of gas and a visible color change of the pH indicator, phenol red. The production of hydrogen sulphide in the medium is indicated by the formation of a black precipitate that will blacken the medium in the butt of the tube.

To facilitate the observation of carbohydrate utilization patterns, TSI Agar contains three fermentative sugars, lactose and sucrose in 1% concentrations and glucose in 0.1% concentration. Due to the building of acid during fermentation, the pH falls. The acid

base indicator Phenol red is incorporated for detecting carbohydrate fermentation that is indicated by

the change in color of the carbohydrate medium from orange red to yellow in the presence of acids. In case of oxidative decarboxylation of peptone, alkaline products are built and the pH rises. This is indicated by the change in colour of the medium from orange red to deep red. Sodium thiosulfate and ferrous ammonium sulfate present in the medium detects the production of hydrogen sulfide and is indicated by the black color in the butt of the tube.

Carbohydrate fermentation is indicated by the production of gas and a change in the colour of the pH indicator from red to yellow. To facilitate the detection of organisms that only ferment glucose, the glucose concentration is one-tenth the concentration of lactose or sucrose. The meagre amount of acid production in the slant of the tube during glucose fermentation oxidizes rapidly, causing the medium to remain orange red or revert to an alkaline pH. In contrast, the acid reaction (yellow) is maintained in the butt of the tube since it is under lower oxygen tension.

After depletion of the limited glucose, organisms able to do so will begin to utilize the lactose or sucrose. To enhance the alkaline condition of the slant, free exchange of air must be permitted by closing the tube cap loosely. If the tube is tightly closed, an acid reaction (caused solely by glucose fermentation) will also involve the slant.

Materials Required

Culture

24 hour typticase soy broth culture.

Media

Triple sugar-iron agar slants.

Equipments

Bunsen burner

Inoculating needle

Test tubes

Marking pen

Media preparation: Triple sugar-iron agar (pH 7.4)

Add 3.0 gram of Beef extract, 3 gram of yeast extract, 15 gram of peptone, 5 grams of protease peptone, 10.0 grams of lactose, 10.0 gram of saccharose, 1.0 gram of glucose, 0.2 gram of ferrous sulphate, 5.0 gram of sodium chloride, 0.3 gram of sodium thiosulphate, 0.024 gram of phenol red and 12 gram of agar and make the mixture up to 1000ml with distilled water.

The Peptone mixture and the Beef and Yeast extracts provide the nutrients essential for growth. Sodium chloride maintains the osmotic balance of the medium. The Bacteriological agar is the solidifying agent.

Procedure

1. Sterilize the inoculating needle in the blue flame of the bunsen burner till red hot and then allowed to cool.
2. From the rack, take the Trypticase soy broth tube containing the 24-48 hour culture, remove the cap and flame the neck of the tube.
3. Using aseptic technique, take the culture of the organism from the TSB (tryptic soy broth) tube with the needle.
4. Again flame the neck of the tube and replace the tube in the test tube rack.
5. Take a sterile TSI slant tube from the rack, remove the cap and flame the neck of the tube.
6. Stab the needle containing the pure culture into the medium, upto the butt of the TSI tube, and then streak the needle back and forth along the surface of the slant.
7. Again flame the neck of the TSI tube, cap it and place it in the test tube rack.
8. Incubate at 37^{o}c for 18 to 24 hours.

Expected Results

Alkaline slant (red) and acid butt (yellow) with or without gas production (breaks in the agar butt):

Only glucose fermentation has occurred. The organisms preferentially degrade glucose first. Since this substrate is present in minimal concentration, the small amount of the acid produced on the slant surface is oxidized rapidly. The peptones in the medium are also used in

the production of alkali. At the butt, the acid reaction is maintained because of the reduced oxygen tension and slower growth of the organisms.

Acid slant (yellow) and acid butt (yellow) with or without gas production:

Lactose or sucrose fermentation has occurred. Since these substances are present in higher concentrations, they serve as substrates for continued fermentative activities with maintenance of an acid reaction in both the slant and the butt.

Alkaline slant (red) and alkaline butt (red) or no change (orange-red) butt:

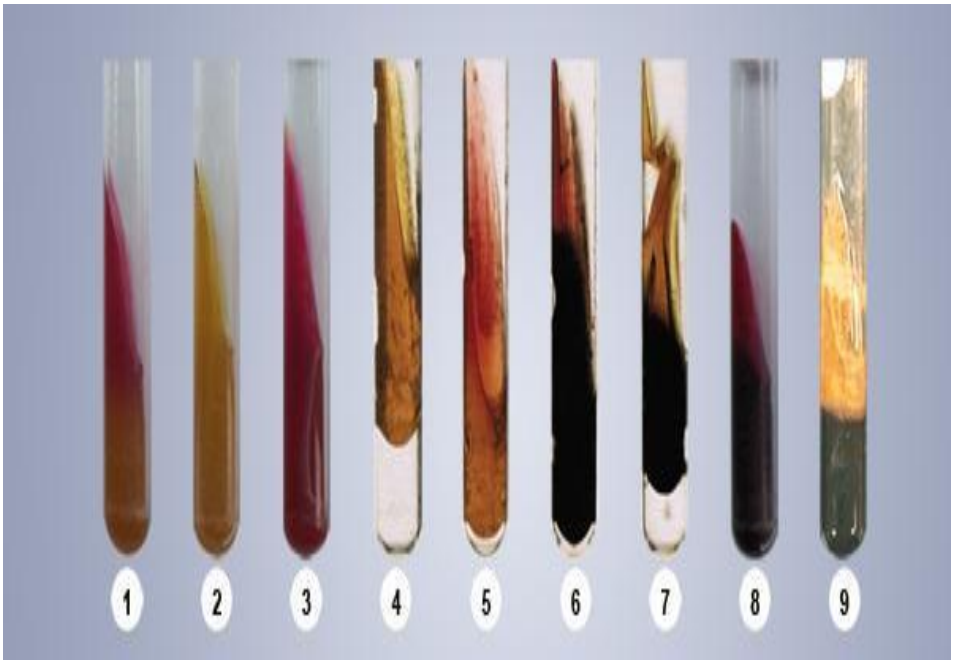
No carbohydrate fermentation has occurred. Instead; peptones are catabolized under anaerobic and /or aerobic conditions resulting in alkaline pH due to production of ammonia. If only aerobic degradation of peptones occurs, the alkaline reaction is evidenced only on the slant surface. If there is aerobic and anaerobic utilization of peptone, the alkaline reaction is present on the slant and the butt.

Hydrogen sulfide (H_2S) production:

Some bacteria utilize thiosulfate anion as a terminal electron acceptor, reducing it to sulfide. If this occurs, the newly-formed hydrogen sulfide (H2S) reacts with ferrous sulfate in the medium to form ferrous sulfide, which is visible as a black precipitate. The blackening of the medium is almost always observed in the butt (bottom) of the medium.

Carbon dioxide (CO_2) production:

It is recognized simply as bubbles of gas between the agar and the wall of the tube or within the agar itself. The carbon dioxide production is sufficient to split the agar into two or more sections. To obtain accurate results, it is absolutely essential to observe the cultures within 18-24 hours following incubation. This will ensure that the carbohydrate substrates have not been depleted and that degradation of peptones yielding alkaline end products has not taken place.



Triple Sugar- Iron Agar Test

S.No	Result (slant/butt)	Symbol	Interpretation
1	Red/Yellow	K/A	Glucose fermentation only, peptone catabolized.
2	Yellow/Yellow	A/A	Glucose and lactose and/or sucrose fermentation.
3	Red/Red	K/K	No fermentation, Peptone catabolized.
4	Yellow/Yellow with bubbles	A/A,G	Glucose and lactose and/or sucrose fermentation, Gas produced.
5	Red/Yellow with bubbles	K/A,G	Glucose fermentation only, Gas produced.
6	Red/Yellow with bubbles and black precipitate	K/A,G,H_2S	Glucose fermentation only, Gas produced, H_2S produced.
7	Yellow/Yellow with bubbles and black precipitate	A/A,G,H_2S	Glucose and lactose and/or sucrose fermentation, Gas produced, H_2S produced.
8	Red/Yellow with black precipitate	K/A,H_2S	Glucose fermentation only, H_2S produced.
9	Yellow/Yellow with black precipitate	A/A,H_2S	Glucose and lactose and/or sucrose fermentation, H_2S produced.

5. ENUMERATION OF MICROORGANISM - TOTAL & VIABLE COUNT

A. ENUMERATION OF BACTERIA USING TOTAL CELL COUNT (DIRECT MICROSCOPIC METHOD)

In the direct microscopic count, a counting chamber consisting of a ruled slide and a cover slip is employed.. It is constructed in such a manner that the cover slip, slide, and ruled lines delimit a known volume. It is constructed in such a manner that the cover slip, slide, and ruled lines delimit a known volume. The number of bacteria in a small known volume is directly counted microscopically and the number of bacteria in the larger original sample is determined by extrapolation.

The Petroff-Hausser counting chamber has small etched squares 1/20 of a millimeter (mm) by 1/20 of a mm and is 1/50 of a mm deep. The volume of one small square therefore is 1/20,000 of a cubic mm or 1/20,000,000 of a cubic centimeter (cc). There are 16 small squares in the large double-lined squares that are actually counted, making the volume of a large double-lined square

1/1,250,000 cc. The normal procedure is to count the number of bacteria in five large double-lined squares and divide by five to get the average number of bacteria per large square. This number is then multiplied by 1,250,000 since the square holds a volume of 1/1,250,000 cc, to find the total number of organisms per cc in the original sample.

If the bacteria are diluted, such as by mixing the bacteria with dye before being placed in the counting chamber, then this dilution must also be considered in the final calculations.

The formula used for the direct microscopic count is:

The number of bacteria per cc
=

The average numbers of bacteria per large double-lined square

X The dilution factor of the large square (1,250,000) X

The dilution factor of any dilutions made prior to placing the sample in the counting chamber, e.g., mixing the bacteria with dye

Methodology

1. Pipette 1.0 ml (see Fig. 8) of the sample of E. coli into a tube containing 1.0 ml of the dye methylene blue. This gives a 1/2 dilution of the sample.

2. Using a Pasteur pipette, fill the chamber of a Petroff-Hausser counting chamber with this 1/2 dilution.

3. Place a coverslip over the chamber and focus on the squares (Fig. 2B) using 400X (40X objective).

4. Count the number of bacteria in 5 large double-lined squares. For those organisms on the lines, count those on the left and upper lines but not those on the right and lower lines. Divide this total number by 5 to find the average number of bacteria per large square.

5. Calculate the number of bacteria per cc as follows:

The formula used for the direct microscopic count

is: The number of bacteria per cc =

The average numbers of bacteria per large double-lined square

X The dilution factor of the large square (1,250,000) X

The dilution factor of any dilutions made prior to placing the sample in the counting chamber, e.g., mixing the bacteria with dye

B. ENUMERATION OF BACTERIA IN A POPULATION: USING SERIAL DILUTIONS AND PLATING TO ESTABLISH VIABLE BACTERIAL CELL COUNT

Scientists use a number of different methods to determine the number of micro-organisms that are present in a given population. This can be accomplished by using the spectrophotometer to measure the optical density of the population, by directly counting the microorganisms using a haemocytometer, or by serial diluting the bacteria and plating the diluted bacteria on media that supports the growth of the micro-organisms. The latter method is somewhat more time consuming, but provides statistically accurate and repeatable results. This method is also the ideal method for enumerating microorganisms in a given population because it only identifies the living organisms in that population.

Microbial counting is useful in the basic sciences and is used determine the number of bacteria present for physiological or biochemical studies. For example, if one knows the number of bacteria present in a culture then one can calculate the amount of protein or DNA that can be isolated from that population. Microbial enumeration is also routinely used in the areas of public

health. Food or water microbiologists test food, milk or water for the numbers of microbial pathogens to determine if these products are safe for human consumption.

We will be using serial dilutions, plating and counting of live bacteria to determine the number of bacteria in a given population. To this end we will make serial dilutions of a solution containing an unknown number of bacteria, plate these bacteria and determine the total number of bacteria in the original solution by counting the number of colony forming units and comparing them to the dilution factor. Each colony forming unit represents a bacterium that was present in the diluted sample. The numbers of colony forming units (CFU's) are divided by the product of the dilution factor and the volume of the plated diluted suspension to determine the number of bacteria per mL that were present in the original solution.

Lab 1. Serial dilutions:

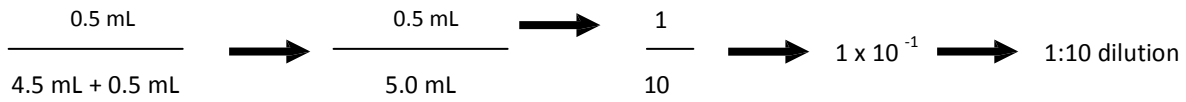
Students should obtain 10 small, sterile test tubes, label the tubes 1 through 10 and then add 4.5 mL of M9 salts to each test tube. M9 salts is a physiological buffered minimal medium that contains inorganic salts but no carbon source. Bacteria will not grow in this media but will remain in a state of stasis until the diluted cells are plated on media containing a carbon source.

The students should pipette 0.5 mL of the original solution into test tube 1. This bacterial suspension should be mixed thoroughly (using the vortexers on each bench) before proceeding to the next step. The students should obtain a clean pipette and withdraw 0.5 mL of the diluted bacterial suspension from the first test tube and pipette that into the second test tube. Continue in this fashion until you have serially diluted the original bacterial suspension into test tube 7. The instructor will show you how to perform this exercise. In test tube 1 you have diluted the

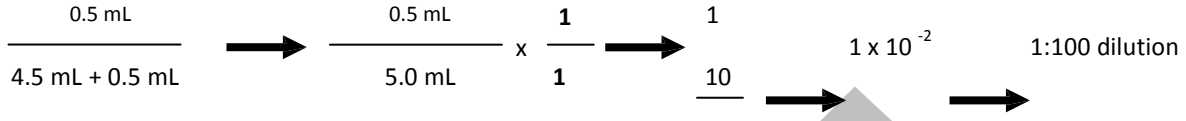
bacteria 10 fold, a 1:10 or 1×10^{-1} dilution, in test tube 5 you have diluted the bacteria from the original tube to obtain a 1×10^{-5} dilution, in test tube 10 you have diluted the bacteria from the original tube to obtain a 1×10^{-10} dilution.

Below is the mathematical reasoning for performing the serial dilutions:

Tube 1 contains 4.5 mL of sterile media; you will add 0.5 mL of the **undiluted** bacterial suspension to yield a total volume of 5.0 mL.



Tube 2 contains 4.5 mL of sterile media; you will add 0.5 mL of the **1:10 diluted** bacterial suspension to yield a total volume of 5.0 mL



Lab 1. Plating the serially diluted cells:

The students should obtain 10 TSA plates from the instructor; these plates should be labeled with your initials and the dilution factor. In this case we will plate the following dilutions: 1×10^{-1}, 1×10^{-2}, 1×10^{-3}, 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, 1×10^{-8}, 1×10^{-9} and 1×10^{-10}.

The students should also obtain a beaker containing a ―hockey stick‖ and pour a small volume of alcohol into the beaker. The hockey stick is used to spread the diluted bacterial suspension evenly over the surface of the plate. **The instructor will demonstrate this process.**

For the cell suspension that will be plated onto the TSA plate labeled **1×10^{-1}**, pipette **0.5 mL** of the diluted suspension from the appropriately diluted test tube onto the surface of the plate. Dip the hockey stick into the alcohol solution and flame the stick until the alcohol has burned off. Do not heat the stick to long; you only need to flame the loop to burn off the alcohol—that will be sufficient for sterilizing the hockey stick. After sterilizing the stick, use the hockey stick to spread the bacterial suspension evenly over the entire surface of the plate. Allow the plate to dry. Continue this process with the remainder of the bacterial dilutions.

Tape all of your plates together and incubate your plates, upside down, at 37^{o}C for 24 hours. During the next period you will count the number of colony forming units for each dilution and calculate the number of bacteria in the original suspension!!!!

Lab 2. Counting colony forming units and calculating the amount of bacteria in the original solution.

The instructor will provide a counter and your plates. For each dilution, count the number of colony forming units on your plates. Typically numbers between 30 and 800 are considered to be in the range where one's data is statistically accurate. If the number of CFUs on your plate are greater than 1000, you may record in your table TNTC (too numerous to count). Alternatively, if your numbers are greater than 1000 AND you have evenly distributed the diluted bacterial suspension on the surface of the plate AND you can discern individual colonies; divide the plate into 4 sectors, count the number of bacteria in one sector and multiply by four. If the number of CFUs on your plate is below 10, record the number of CFUs, but do not use this

in your calculations. T=Trial

Dilution factor	**Number of bacterial colonies (CFUs)**									**Avg # CFU**	**Avg # bacteria/ml**
	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**		
$1:10^{-1}$											
$1:10^{-2}$											
$1:10^{-3}$											
$1:10^{-4}$											
$1:10^{-5}$											
$1:10^{-6}$											
$1:10^{-7}$											
$1:10^{-8}$											
$1:10^{-9}$											
$1:10^{-10}$											

Calculating the number of bacteria per mL of serially diluted bacteria:

To calculate the number of bacteria per mL of diluted sample one should use the following equation:

$$\frac{\text{Number of CFU}}{\text{Volume plated (mL) x total dilution used}} \rightarrow \frac{\text{Number of CFU}}{\text{mL}}$$

For example, if for the 1x 10^{-8} dilution plate you plated **0.1 mL** of the diluted cell suspension and counted 200 bacteria, then the calculation would be:

200/0.1 mL x 10^{-8} or 200/10^{-9} or 2.0 x 10^{11} bacteria per mL.

6. DETERMINATION OF BACTERIAL CELL SIZE BY MICROMETRY

Objective

To measure the dimensions of microorganisms with the help of a microscope.

Theory

Microorganisms are microscopic objects that are visible only with the help of a microscope. Sometimes it is necessary to measure its dimensions (length breadth and diameter) for its identification process. But, determination of the size of a microorganism is not an easy process. Micrometry refers to the measurement of dimensions of the desired microorganisms under a microscope which uses two micro-scales known as micrometers‘. At first, the diameter of the microscopic field must be established with the help these micrometers namely ocular micrometer and stage micrometer. Ocular micrometer with microscopic graduations etched on their surfaces is a circular glass disc that fits into the circular shelf inside the eyepiece of the microscope. It has

100 equally spaced divisions marked as 0 to 10. Depending on the objective being used, the distance between these graduations will vary that determines the size of the field. The other micrometer, stage micrometer is clipped to the stage of the microscope. In the centre of the stage micrometer a known 1mm distance is etched into100 equally spaced divisions making each division equals 0.01 mm or 10 µm.

Calibration

With respect to the required objective, the graduations on the ocular microscope are calibrated against the standard graduations on the stage micrometer. Hence the graduations on both micrometers gets superimposed each other.

Calibration steps

Rotate the ocular lens.

Determine the number of ocular divisions per known distance on the stage micrometer.

Materials

Microscope
Ocular micrometer
Stage micrometer
Millimeter ruler
Prepared slide

Procedure

1. Place a stage micrometer on the microscope stage, and using the lowest magnification (4X), focus on the grid of the stage micrometer.
2. Rotate the ocular micrometer by turning the appropriate eyepiece. Move the stage until you superimpose the lines of the ocular micrometer upon those of the stage micrometer. With the lines of the 2 micrometers coinciding at one end of the field, count the spaces of each micrometer to a point at which the lines of the micrometers coincide again.
3. Since each division of the stage micrometer measures 10 micrometers, and since you know how many ocular divisions are equivalent to 1 stage division, you can now calculate the number of micrometers in each space of the ocular scale.
4. Repeat for 10X and 40X, and 100X. Record your calculations below:

Microscope #	
Value for each ocular unit at 4X	
Value for each ocular unit at 10X	
Value for each ocular unit at 40/45X	
Value for each ocular unit at 100X	

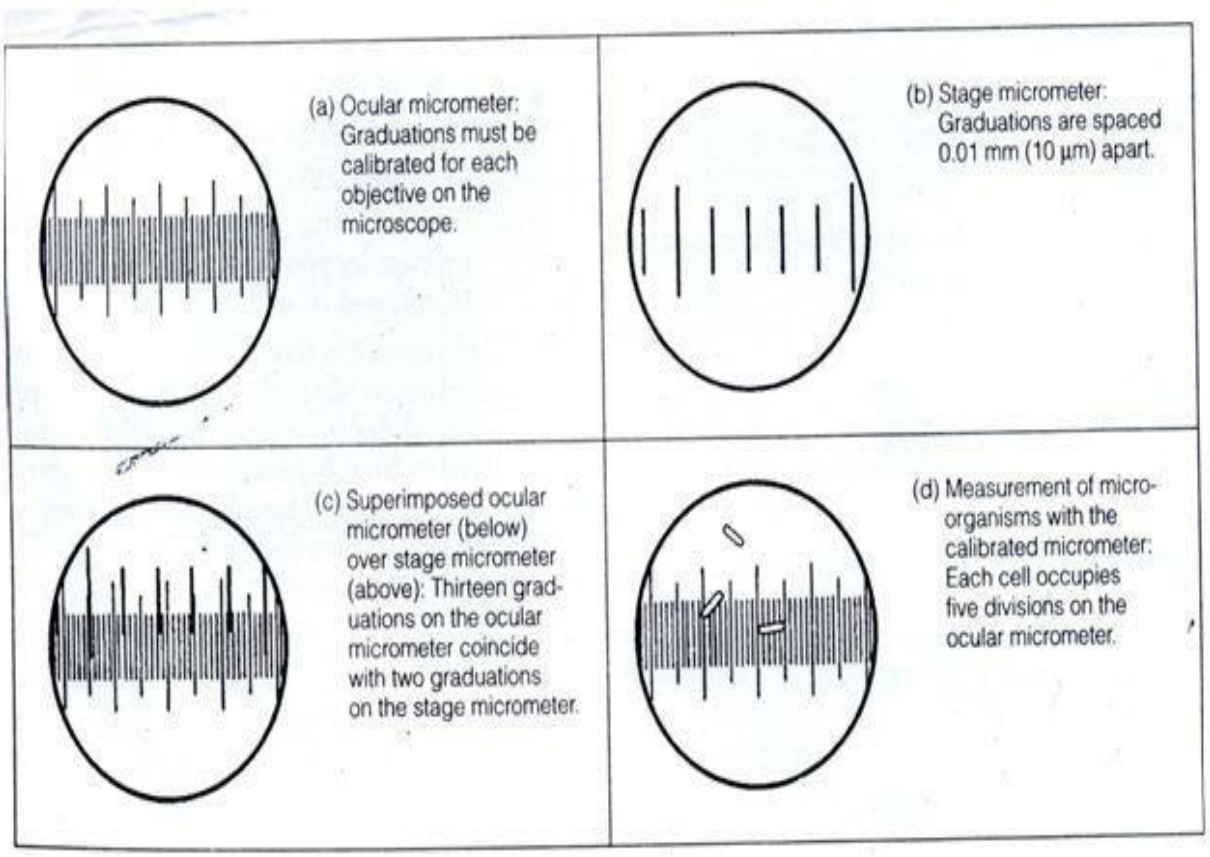
Using the stage micrometer, determine the smallest length (in microns) that can be resolved with each objective. This is the measured limit of resolution for each lens. Compare this value to the theoretical limit of resolution, calculated on the basis of the numerical aperture of the lens and a wavelength of 450 nm (blue light). Using the calculated values for your ocular micrometer, determine the dimensions of the letter ―e‖ found on your microscope slide. Use a millimeter ruler to measure the letter ―e‖ directly and compare it with the calculated values obtained through the microscope.

To measure an object seen in a microscope, an ocular micrometer serves as a scale or rule. This is simply a disc of glass upon which equally spaced divisions are etched. The rule may be divided into 50 subdivisions, or more rarely, 100 subdivisions. To use the ocular micrometer, calibrate it against a fixed and known ruler, the stage micrometer. Stage micrometers also come in varying lengths, but most are 2-mm long and subdivided into 0.01-

mm (10-micrometer) lengths. Each objective will need to be calibrated independently. To use, simply superimpose the ocular micrometer onto the stage micrometer and note the relationship of the length of the ocular to the stage micrometer. Note that at different magnifications, the stage micrometer Notes changes, but the ocular micrometer is fixed in dimension. In reality, the stage micrometer is also fixed, and what is changing is the power of the magnification of the objective.

Calculation

Calibration factor for One division on ocular micrometer (in mm) = (Known distance between two lines on stage micrometer) /(Number of divisions on ocular micrometer)



After calibration, the ocular micrometer measures the size of various microbes including its length, breadth, and diameter. First count the number of spaces occupied by the organism. Then

multiply this number by the calculated calibration factor. This value indicates the length of the organism. It can also be used to find out organism‘s breadth and diameter.

Application

Helps to determine the sizes of different microorganisms including bacteria, protozoa and yeast.